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Misacylation of tRNA with methionine in *Saccharomyces cerevisiae*

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ABSTRACT

Accurate transfer RNA (tRNA) aminoacylation by aminoacyl-tRNA synthetases controls translational fidelity. Although tRNA synthetases are generally highly accurate, recent results show that the methionyl-tRNA synthetase (MetRS) is an exception. MetRS readily misacylates non-methionyl tRNAs at frequencies of up to 10% in mammalian cells; such mismethionylation may serve a beneficial role for cells to protect their own proteins against oxidative damage. The *Escherichia coli* MetRS mismethionylates two *E. coli* tRNA species *in vitro*, and these two tRNAs contain identity elements for mismethionylation. Here we investigate tRNA mismethionylation in *Saccharomyces cerevisiae*. tRNA mismethionylation occurs at a similar extent *in vivo* as in mammalian cells. Both cognate and mismethionylated tRNAs have similar turnover kinetics upon cycloheximide treatment. We identify specific arginine/lysine to methionine-substituted peptides in proteomic mass spectrometry, indicating that mismethionylated tRNAs are used in translation. The yeast MetRS is part of a complex containing the anchoring protein Arc1p and the glutamyl-tRNA synthetase (GluRS). The recombinant Arc1p–MetRS–GluRS complex binds and mismethionylates many tRNA species *in vitro*. Our results indicate that the yeast MetRS is responsible for extensive misacylation of non-methionyl tRNAs, and mismethionylation also occurs in this evolutionary branch.

INTRODUCTION

Translational fidelity, which is critical for cell survival, depends on the incorporation of the correct amino acid to its transfer RNA (tRNA). Aminoacyl-tRNA synthetases (aaRSs) aminoacylate tRNAs with their cognate amino acids. Despite the high fidelity of catalytic and editing domains in aaRSs, misacylation can occur when the aaRS catalyzes the aminoacylation of a cognate tRNA with a non-cognate amino acid (1). aaRS can also misacylate tRNAs through catalyzing the aminoacylation of a non-cognate tRNA with a cognate amino acid. Misacylated tRNAs that are used in translation produce mutant proteins. However, mistranslation at low levels is not always detrimental to cells and in some cases is tolerated or may even be beneficial in stress response (2,3).

A recent study has provided evidence that tRNA misacylation with methionine is actively regulated in mammalian cells and tRNA mismethionylation may provide a benefit to cells under oxidative stress (4). Previous findings show that genetically encoded methionine residues can protect proteins against reactive oxygen species by oxidation of methionine residues on the surface or near active sites of proteins (5,6). Mismethionylated tRNAs can extend this protective function by substituting certain non-methionine residues in proteins at strategic locations. For this function, methionine substitution through mismethionylated tRNAs is likely to occur at solvent-exposed residues on the surface or near the active site of target proteins; such residues more commonly have charged or polar side chains.

tRNA mismethionylation occurs in both prokaryotes and eukaryotes. The recombinant, purified *E. coli* methionyl-tRNA synthetase (MetRS) mismethionylates two *E. coli* tRNAs *in vitro*, one coding for a charged amino acid, $tRNA^{Arg}_{CCU}$, and the other for a polar amino acid, $tRNA^{Thr}_{CGU}$ (7). In mammalian cells, MetRS is part of an 11 protein complex, eight of which are aaRSs (8,9). The affinity purified human complex containing MetRS can mismethionylate two human tRNAs coding for a charged amino acid, $tRNA^{Lys}_{CUU}$ and $tRNA^{Lys}_{UUU}$ (4). In mammalian cells, mismethionylation occurs at a basal level of ~1%, which includes tRNAs coding for several charged amino acids. This level of

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mismethionylation increases to up to 10% under innate immune activation and chemically triggered oxidative stress (4).

Here we investigate tRNA misacylation with methionine in baker's yeast, *Saccharomyces cerevisiae*, both *in vivo* and *in vitro*. *Saccharomyces cerevisiae* is evolutionary distant from mammals and bacteria, so studies of yeast broaden the evolutionary reach of this unusual behavior in altering the aminoacylation and translational fidelity. Using tRNA microarrays and classical filter retention assays, we identified many mismethionylated tRNAs in *S. cerevisiae*. The extent of misacylated tRNAs is dependent on yeast growth conditions. Mismethionylated tRNAs show similar *in vivo* utilization kinetics as correctly charged $tRNA^{Met}$s, and several misacylated peptides are detected by mass spectrometry, indicating that they are used in translation. The yeast MetRS is part of a three protein complex that includes a general tRNA-binding protein, Arc1p and the glutamyl-tRNA synthetase [GluRS, (10)]. Previous reports have shown that the anticodon is not sufficient for tRNA binding to the yeast MetRS; efficient aminoacylation with methionine requires primary, secondary and tertiary determinants in the tRNA (11–13). The Arc1p protein binds to the MetRS and GluRS by N-terminal interactions and to tRNA by C-terminal interactions, which facilitate tRNA binding to the MetRS and GluRS for aminoacylation with methionine and glutamic acid, respectively (10). However, since Arc1p binding to tRNA is non-specific, tRNA binding to the MetRS determines the specificity of aminoacylation (10,14). We examine tRNA binding to the recombinant Arc1p-GluRS-MetRS (AME) complex using tRNA microarrays and show that AME can bind almost all yeast tRNAs, consistent with the previous reports. We also show that AME extensively mismethionylates many tRNAs *in vitro*. Our results suggest that MetRS being a part of a multi-protein complex provides eukaryotes with another mechanism of tRNA mismethionylation by allowing many tRNAs to bind to the MetRS for misacylation with methionine.

MATERIALS AND METHODS

Yeast strains

Yeast strains used in this study were WY798 (*MATα URA3 LEU2 TRP1*) (15), BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) and ΔArc1 (*MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 arc1Δ*). BY4742 and ΔArc1 were purchased from Open Biosystems. Yeast strains were grown on YPDA medium at 30°C. Single colonies were grown overnight in synthetic complete (SC) media. For stationary phase experiments, these cultures were grown to OD_{600} 4–8. For mid-log phase experiments, the overnight cultures were diluted to OD_{600} 0.1 and grown to OD_{600} 0.4–0.5.

Pulse-labeling

^{35}S-Met pulse labeling of yeast cells was adapted from established procedures with minor modifications. Briefly, yeast cells were starved for methionine by spinning down and resuspending in an equal volume of SC–Met media 1 h prior to pulse labeling to maximize ^{35}S-signal. After pelleting, yeast cells were resuspended in 300 µl of pulse labeling media consisting of 0.02 mCi/OD_{600} ^{35}S-Met (Perkin-Elmer, Boston, MA) in SC–Met. OD_{600} 12.5 cells were typically sufficient to yield ~100 µg RNA. Pulse labeling proceeded at 30°C for 1 or 8 min. For chase experiments, 300 µl of SC–Met supplemented with 1 mg/mL fresh methionine, and 200 µg/mL cycloheximide, where appropriate, was added after the pulse period and incubated at 30°C for 1 min. Reactions were stopped by addition of 300 µl ice cold 0.3 M sodium acetate/acetic acid buffer with 10 mM EDTA, pH 4.8, and submersion in ice, after which cells were further rinsed twice with the same buffer. For cycloheximide treatments, 200 µg/mL cycloheximide was maintained in the acetate/EDTA buffer solution throughout washes and lysis to maintain translational arrest.

RNA isolation

For *in vitro* experiments, total RNA was isolated from yeast grown to stationary phase overnight in YPDA medium, pelleted, resuspended in 300 µl 0.3 M KCl, 50 mM KOAc, and transferred to a tube containing 300 µl acetate-saturated phenol-$CHCl_3$, pH 4.8 and 0.5 mm acid-treated glass beads. The sample was vortexed three times by alternating vortexing for 1 min and incubating on ice. The sample was then spun at 14 000 rpm for 15 min at 4°C, transferred to a new tube containing 300 µl acetate-saturated phenol-$CHCl_3$, pH 4.8, and vortexed for an additional 1 min. The sample was spun at 14 000 rpm for 10 min at 4°C, and the aqueous layer was transferred to a clean tube, ethanol precipitated twice, and resuspended in 10 mM Tris, pH 7.5, 1 mM EDTA.

Following pulse labeling *in vivo*, total RNA was isolated from yeast by transferring the sample to a clean tube containing 300 µl acetate-saturated phenol-$CHCl_3$, pH 4.8, and 1 Retsch 7 mm stainless steel ball and vortexing at room temperature for 30 min. The sample was then spun at 14 000 rpm for 15 min at 4°C and followed the remaining procedure as in the *in vitro* experiments. Once resuspended in 10 mM Tris, pH 7.5, 1 mM EDTA, the RNA was again spun at 14 000 rpm for 15 min at 4°C and transferred to a clean tube.

Purification of the Arc1p-GluRS-MetRS complex

A plasmid overexpressing the AME complex under IPTG control was transformed into BL21 DE3 *E. coli* cells. The cells were grown in LB with 100 mg/L ampicillin until OD_{600} 0.6, and then overexpression was induced with 0.2 mM IPTG at 37°C. Expression continued for 4 h, and the cells were then harvested. Cells were lysed in lysing buffer (50 mM K-HEPES, pH 7.6, 30 mM NaCl, 5 mM β-mercaptoethanol) in the presence of protease inhibitors and 2000 U DNase per 50 mL extract. Following centrifugation, the complex was purified by FPLC by elution from a Ni-NTA column using an imidazole gradient. The purification buffers contained 50 mM K-HEPES, pH 7.6, 150 mM NaCl, 5% glycerol, 10 mM

BME, and 20 mM or 500 mM imidazole. The complex eluted around 300 mM imidazole.

Gel filtration of the Arc1p–GluRS–MetRS complex

The affinity purified AME complex was passed through a Superdex 200 column at 4°C to analyze by gel filtration using the buffer containing 20 mM Tris, pH 7.4, 30% glycerol, 2 mM DTT and 1 M NaCl.

In vitro transcription

Saccharomyces cerevisiae $tRNA^{Met}_{CAU}$, $tRNA^{Glu}_{CUC}$, $tRNA^{Glu}_{UUC(12)}$ and $tRNA^{Glu}_{UUC(1)}$ sequences were obtained from the genomic tRNA database (16). Mutants 1-3 were created by swapping nucleotides from the $tRNA^{Glu}_{CUC}$ and $tRNA^{Glu}_{UUC(12)}$. For $tRNA^{Met}_{CAU}$, the transcript was made by *in vitro* transcription of overlapping oligonucleotides and purified as described previously (17,18).

All mature $tRNA^{Glu}$ start with U at the 5′-position. Since T7 RNA polymerase transcription works poorly with U-starting RNA, the three $tRNA^{Glu}$ transcripts and mutants 1-3 were first transcribed similarly as the $tRNA^{Met}$ transcript but with a 5′-leader sequence of 5′-gggacaaata-$tRNA^{Glu}$. These transcripts were then cleaved with *Bacillus subtilis* RNase P holoenzyme to obtain the appropriate tRNA sequences. The cleavage was performed by first reconstituting the *B. subtilis* RNase P holoenzyme (19). The final buffer concentration of the reconstituted holoenzyme was 50 mM Tris-HCl, pH 8, 18 mM $MgCl_2$, 0.2 M NH_4Cl. The holoenzyme was reconstituted by first mixing P RNA with water and Tris–HCl, pH 8, and heating at 85°C for 2 min, then at room temperature for 3 min, followed by adding $MgCl_2$ and incubating at 50°C for 5 min, and finally adding equal moles of P protein and NH_4Cl and incubating at 37°C for 5 min. The transcription mixture was then incubated with the reconstituted *B. subtilis* RNase P at 37°C for 5 min. Cleavage was stopped with 15 mM EDTA, and the cleaved transcription mixture was ethanol precipitated and purified by denaturing PAGE.

Filter-based aminoacylation reactions

Filter-based aminoacylation reactions with methionine were performed at 30°C in 20 mM K-HEPES (pH 7.2), 100 μM methionine, 10 mM $MgCl_2$, 5 mM DTT, 4 mM ATP, 150 mM NH_4Cl, 0.1 mM EDTA, 0.5 μCi/mL L-[^{35}S] methionine, 0 or 2.5 μM tRNA transcripts and 0.1 μM AME enzyme. Filter-based aminoacylation reactions with glutamic acid were performed at 30°C in 100 mM K-HEPES (pH 7.2), 100 μM glutamic acid, 10 mM $MgCl_2$, 10 mM DTT, 2 mM ATP, 30 mM KCl, 50 μM L-[^{3}H] glutamic acid, 0 or 2.5 μM tRNA transcripts and 0.1 μM AME. Filters were counted on a Perkins–Elmer scintillation counter. For kinetics experiments, aminoacylation reactions were performed with up to 15 μM transcripts.

In vitro aminoacylation reactions

In vitro aminoacylation of tRNA for microarray analysis was performed at 30°C for 6 or 20 min in 20 mM K-HEPES (pH 7.2), 100 mM NH_4Cl, 0.1 mM Na-EDTA, 2 mM ATP, 1.5 mM $MgCl_2$, 2.5 mM DTT, 1 mCi/mL L-[^{35}S]methionine (Perkins–Elmer, Boston, MA), 0.4 mg/mL gel-purified total yeast tRNA and 0.5 μM purified AME enzyme.

5′-^{32}P-tRNA binding to Arc1p–GluRS–MetRS complex

Total tRNA was gel-purified from total RNA isolated from yeast at pH 4.8. The purified tRNA was dephosphorylated with calf intestinal phosphatase in 50 mM Tris, pH 8, 0.1 mM EDTA, extracted from phenol/$CHCl_3$ and ethanol precipitated. The tRNA was 5′-^{32}P-labeled with T4 PNK and renatured. Binding experiments contained 10 pmol AME with excess (40 pmol total) renatured tRNA doped with 5′-^{32}P-labeled tRNA in 0.1 M K-HEPES, pH 7.2, 1.5 mM Mg^{2+} and 0.1–0.4 M KCl. The binding mixture was incubated at 30°C for 10 min and then added to Genscript Ni^{2+}-MagBeads following the Genscript protocol 2.1.2 for purification of polyhistidine-tagged proteins under native conditions. The wash and elution buffers were the same as used in the Ni-NTA purification of the AME complex. The eluted tRNA was ethanol precipitated and analyzed by tRNA microarray.

Microarray analysis

Hybridization to microarrays and controls using radioactive detection on a Genomic Solutions Hyb4 station has been described previously (4). Mismethionylation and tRNA binding to AME for yeast was assessed with manually printed arrays containing 40 nuclear and 24 mitochondrial probes for *S. cerevisiae* and 31 probes for *E. coli* as controls. The arrays contained eight replicates for each probe. Experiments with ^{35}S-Met detection used 20 μg total RNA per array. Signals were quantified using Fuji Imager software.

Mass spectrometry analysis

Mass spectrometry data and FASTA sequences for nine abundant yeast proteins (ADH1, CDC19, ENO1, ENO2, FBA1, PDC1, PGK1, TDH2 and TDH3) were obtained from Geiler-Samerotte *et al.* (20) and were analyzed by MaxQuant (21). Additional FASTA sequences were created for each protein with one methionine substitution at each lysine and arginine residue. As trypsin-digested peptides are cleaved at Lys and Arg residues, the MaxQuant data were analyzed for longer peptides representing a methionine misincorporation at the lysine and arginine residues.

RESULTS

tRNA mismethionylation in yeast cells

To determine if tRNAs are misacylated in *S. cerevisiae*, we chose to work first with the *S. cerevisiae* strain 798, a fully prototrophic strain whose tRNA abundance and charging

characteristics have been characterized previously (15). We detected tRNAs that are either correctly or incorrectly aminoacylated with methionine after pulse labeling with ^{35}S-Met using arrays containing probes for all cytosolic and mitochondrial tRNAs of *S. cerevisiae*. The array includes eight repeats each of 40 cytosolic and 24 mitochondrial *S. cerevisiae* tRNA probes. In addition, the array includes eight repeats each of 1 blank control and 31 *E. coli* tRNA probes, which serve as negative controls. The probe sequences used were identical to those described previously to measure tRNA charging in yeast and *E. coli* (22,23).

We observed ^{35}S-signals from numerous yeast tRNA probes for both methionyl and non-methionyl-tRNAs (Figure 1A). The most intense signals were derived from both cytosolic $tRNA^{Met}$s and both mitochondrial $tRNA^{Met}$s, as expected. The strongest signal was derived from cytosolic elongator $tRNA^{Met}_e$. Signals from mitochondrial tRNAs were weaker than $tRNA^{Met}_e$, presumably due to the significantly lower abundance of these tRNAs. Unexpectedly, signal from cytosolic initiator $tRNA^{Met}_i$ was much weaker than that of $tRNA^{Met}_e$, even though the abundance and charging levels of $tRNA^{Met}_i$ and $tRNA^{Met}_e$ should be similar according to previous studies (23,24). Varying pulse labeling time from 1 to 8 min did not markedly change this behavior (data not shown). This result is significantly different from mammalian studies where similar levels for ^{35}S-charging signals were observed for $tRNA^{Met}_i$ and $tRNA^{Met}_e$ (4). At this time, we do not understand the reasons for the low ^{35}S-detected charging levels of $tRNA^{Met}_i$ in pulse labeling. One possible explanation is that yeast cells may use distinct intracellular methionine pools to charge $tRNA^{Met}_i$ and $tRNA^{Met}_e$; our result would be consistent with methionine used for $tRNA^{Met}_e$ coming from immediate Met uptake and methionine used for $tRNA^{Met}_i$ coming from Met obtained or *de novo* synthesized at earlier times.

We performed a series of controls to ensure that the majority of the ^{35}S-signals present in non-methionyl-tRNAs are derived from mismethionylated tRNAs (Figure 1B) as was done previously for the mammalian misacylation study (4). To rule out signals due to cross-hybridization, we added excess oligonucleotides complementary to all cytosolic and mitochondrial $tRNA^{Met}$ to the hybridization mixture. All signals from Met-tRNAs could be eliminated with little change in the signal intensity from non-Met-tRNAs. To rule out signals due to peptidyl-tRNAs with an N-terminal ^{35}S-methionine, we treated the RNA sample with aminopeptidase-M before array hybridization. Most signals from non-Met-tRNAs remain after aminopeptidase treatment (Figure 1C). Post-transcriptional thio-modifications of tRNA may be radio-labeled via catabolism of ^{35}S-Met. To distinguish signals due to post-transcriptional thio-modifications of tRNA, we hydrolyzed the labile aminoacyl bond of the charged tRNA sample at pH 9 prior to array hybridization to remove all ^{35}S-signal due to methionine charging. Yeast tRNAs known to contain thio-modifications at the wobble position of the anticodon include $tRNA^{Lys}_{UUU}$, $tRNA^{Glu}_{UUC}$, $tRNA^{Gln}_{UUG}$ (5-methoxycarbonylmethyl-2-thiouridine or mcm^5s^2U), $tRNA^{Arg}_{UCU}$ and $tRNA^{Thr}_{IGU}$ (2-thio-U or s^2U (25)). Signals from these five tRNAs were readily detected after deacylation. Surprisingly, signals from several other tRNAs were also present after deacylation, including $tRNA^{Lys}_{CUU}$, $tRNA^{Ile}_{IAG,}$ and $tRNA^{Thr}_{UGU}$; these tRNAs do not contain known thio-modifications (Figure 1C). It remains to be determined whether these deacylation resistant ^{35}S-signals are also derived from thio-modifications in these tRNA.

We performed one additional control to ensure that misacylation observed in yeast is not caused by methionine starvation in the standard pulse-labeling protocol (Figure 1D). In the standard pulse-labeling experiments, yeast cells were first starved for methionine for 1 h before the addition of ^{35}S-Met. This step decreases the intracellular pools of cold Met, resulting in increased specific activity of Met in labeled protein or RNA. This step was not necessary for mammalian cells because they are intrinsically auxotrophic for Met, and the intracellular pools of cold Met is much lower. Misacylation still occurred when yeast had not been starved prior to pulse labeling, although signals for both Met and non-Met-tRNAs were substantially reduced.

A summary of the misacylation result is shown in Figure 1C. Approximately two-thirds of all non-Met-tRNA probes show detectable ^{35}S-signals. Alkaline deacylation prior to array hybridization removed signals from ~70% of these probes, suggesting that the majority of these signals are derived from mismethionylation. No mismethionylation was detected for all mitochondrial tRNAs. At the stationary phase, the cumulative extent of misacylation is over 10% relative to all Met-$tRNA^{Met}$ signals. Since the ^{35}S-signal for $tRNA^{Met}_i$ is significantly lower in yeast than in mammalian cells and the misacylation extent is normalized to ^{35}S-signals of all Met-tRNAs, this level of mismethionylation in yeast is comparable to the highest level observed in mammalian cells.

We determined whether tRNA mismethionylation depends on cell growth conditions in *S. cerevisiae* (Figure 2A and B). The growth state of yeast is known to impact numerous cellular factors, including gene expression, metabolic rate and oxidative stress load (26–28). In addition, the stationary phase is thought to impose an oxidative stress relative to the mid-log phase (29). Yeast 798 strain was grown to either mid-log (OD_{600}~0.5) or stationary (OD_{600}~4–8) phase and pulse-labeled with ^{35}S-Met. Mismethionylation was observed under both conditions; however, a greater extent of misacylation occurred in the stationary phase than in the mid-log phase (Figure 2B). This result indicates that the extent of misacylation depends on the yeast growth phase. In mammals, mismethionylation is increased upon innate immune or chemically triggered oxidative stress.

In vitro experiments using HeLa multi-synthetase complexes containing MetRS suggest that the higher order structure of MetRS may play a role in regulating misacylation (4). Yeast MetRS is part of a three protein complex, including the GluRS and a general tRNA binding protein Arc1p. We tested a potential role of

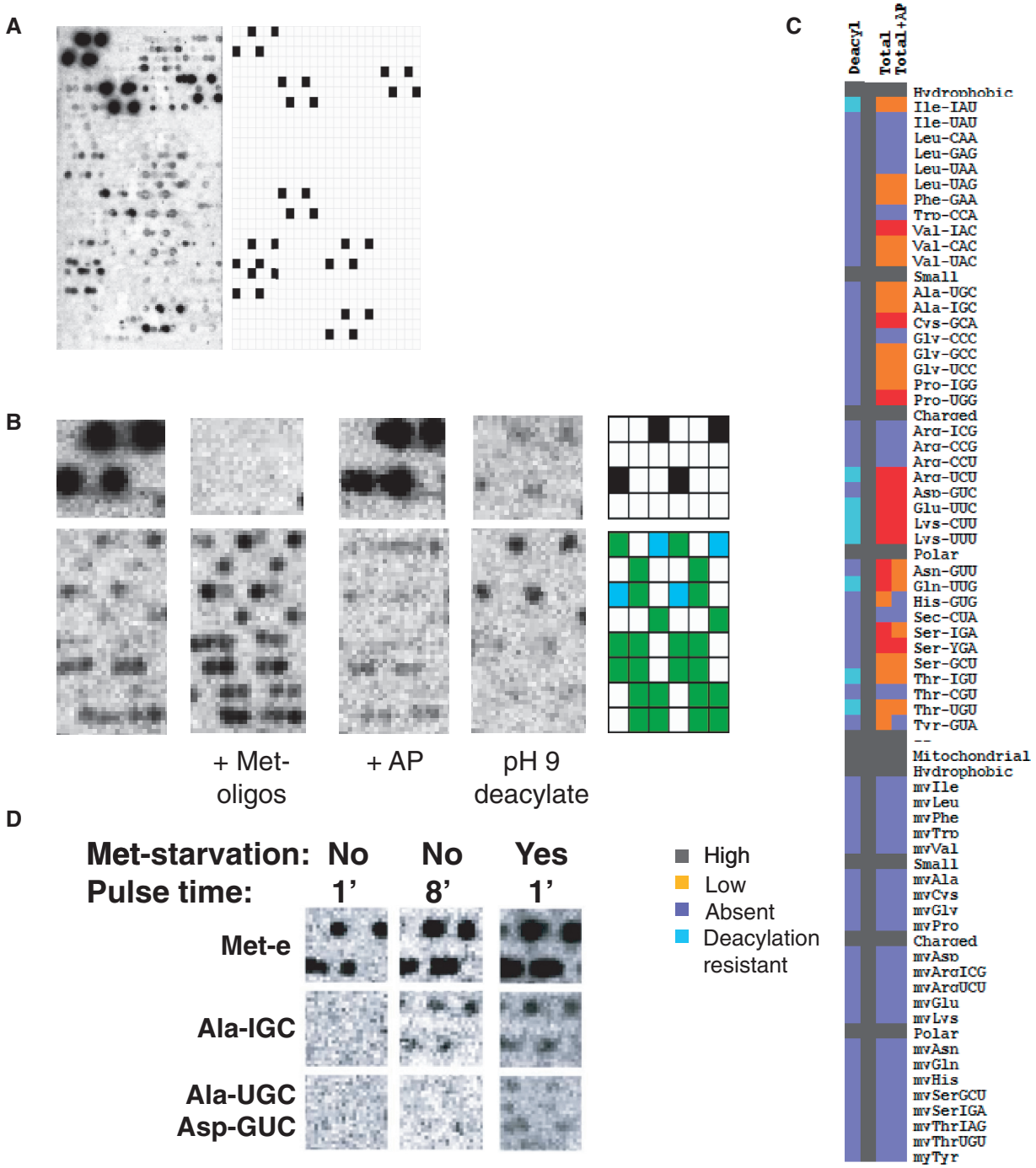


Figure 1. tRNA misacylation with methionine in yeast cells. The full array is shown in panel A. For easier viewing of results, only three array blocks containing the probes of Met_e-tRNA and three examples of the misacylated tRNAs, Ala-IGC, Ala-UGC, and Asp-GUC, are shown in panels B and D and in Figures 2 and 3. (**A**) RNA from the ^{35}S-Met pulse-labeled stationary phase *S. cerevisiae* strain 798 was hybridized to a microarray showing many potentially misacylated tRNAs. Strain 798 is a fully prototrophic strain. All Met-tRNA probes (two for cytosolic and two for mitochondrial) are shown as black squares in the array layout. (**B**) Array controls for mismethionylation include cross-hybridization with excess free Met-tRNA probes (+Met probes), peptidyl-tRNA following treatment with aminopeptidase M (+AP), and thio-modification following deacylation (pH 9 deacylation). In this selected array view, the four strong spots remaining correspond to $tRNA^{Arg}_{UCU}$ whose mouse homolog contains a known thio-modification. On the array layout, black = Met-e; green = non-Met yeast tRNAs; cyan = yeast $tRNA^{Arg}_{UCU}$. (**C**) Semi-quantification of misacylation results with and without aminopeptidase treatment. Many cytosolic tRNAs are misacylated, but no misacylation for mitochondrial tRNA was observed. Signals from the deacylation-resistant tRNAs are shown in cyan on the left. At least three of these contain known thio-modifications. (**D**) Misacylation is not exclusively caused by the initial Met starvation in the standard pulse-labeling protocol. ^{35}S-Met pulse labeling of unstarved cells results in much lower signals, but ^{35}S-Met labeling of non-$tRNA^{Met}$s is still detectable.

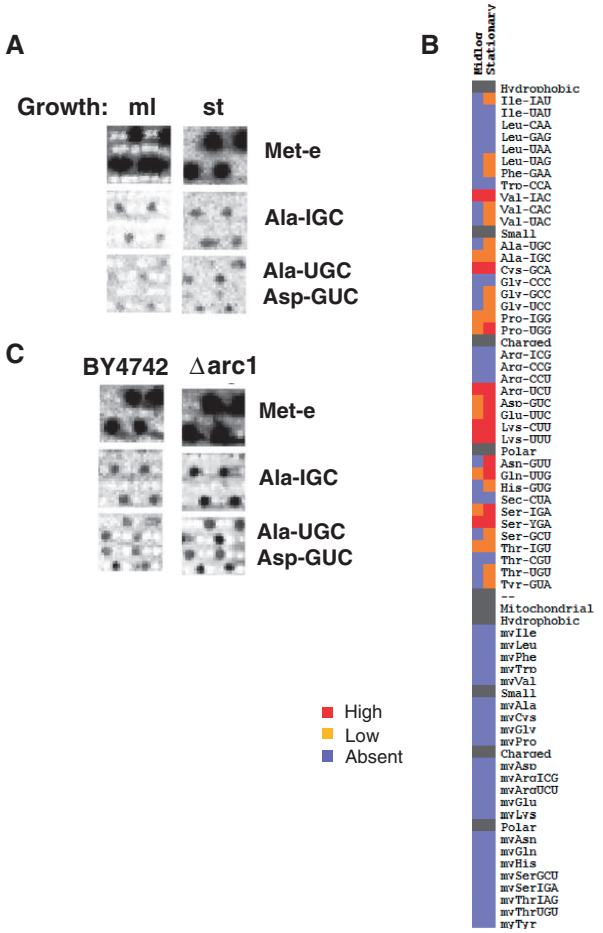


Figure 2. Growth condition dependence of tRNA mismethionylation. (**A**) Signals from mid-log or stationary phase cells from strain 798 show increased mismethionylation at the stationary phase. (**B**) Semi-quantitative comparison. The total amount of mismethionylation increased by ~2-fold from mid-log to stationary. (**C**) Mismethionylation does not require the generic tRNA binding protein Arc1p in the AME complex. Both BY4742 and its isogenic ΔArc1 strain show similar extent of mismethionylation.

Arc1p in tRNA mismethionylation by performing pulse-labeling experiments with an *Arc1* null yeast strain. For this purpose, we switched to yeast cells in the BY4742 background, where the wild-type and the isogenic Δ*Arc1* strains are readily available. No difference in misacylation relative to the wild-type BY4742 strain was detected (Figure 2C). This result suggests that the MetRS alone may be able to methionylate non-methionyl tRNAs *in vivo*.

Aminoacylation of tRNA does not guarantee its use in translation. At least in bacteria, the utilization of an aminoacyl-tRNA depends upon a compromise of specific tRNA and their charged amino acid interactions with the elongation factor and on the ribosome (30,31). Very little is known how yeast elongation factor (eIF1α) and yeast ribosome choose how misacylated tRNA is utilized in translation. We performed one experiment using cold chase and cycloheximide to test whether mismethionylated tRNAs are likely used in translation in yeast (Figure 3). Cycloheximide inhibits ribosome elongation and is widely used in cellular studies of protein synthesis. To examine the kinetics of turnover of mismethionylated tRNAs, cells were first pulse-labeled with ^{35}S-Met, followed by a rapid cold chase of a large excess of non-radioactive Met in the absence and presence of cycloheximide. The resulting ^{35}S-labeled tRNAs were then examined by microarrays. In the absence of cycloheximide, signals from both Met-tRNAs and non-Met-tRNAs were reduced by ~40-fold, suggesting that both types of charged tRNAs are turned over with similar kinetics in cells. In the presence of cycloheximide, the amount of ^{35}S-labeled tRNAs for the 798 strain is reduced by <1.8-fold for Met-tRNAs and non-Met-tRNAs (Figure 3C). This result shows that inhibition of translation also inhibits the turnover kinetics of mismethionylated tRNAs to a similar extent as the turnover of correctly charged Met-tRNAs, consistent with mismethionylated tRNAs being used in translation in yeast cells.

To determine that misacylated tRNAs are indeed used in translation in yeast cells, we analyzed mass spectrometry data from Geiler-Samorette *et al.* (20) using MaxQuant (21). We chose to analyze the peptides for some of the most abundant yeast proteins, including nine proteins involved in glycolysis and fermentation (Figure 4A). The proteins had been trypsin-digested, which cleaves the peptides at Lys and Arg residues, prior to mass spec analysis. Since lysyl- and argininyl-tRNAs are two of the misacylated tRNA species, we chose to look for longer peptides representing methionine misincorporation at Lys and Arg residues. We found low abundant peptides representing misincorporation of methionine at both Lys and Arg residues in seven of these nine proteins at a frequency of 0.66% of all observed peptides for these proteins. Example spectra are shown in Figure 4B for a wild-type and its mistranslated peptide from pyruvate kinase, CDC19. The Arg codon at this misincorporated position in CDC19 is AGA. AGA is read by $tRNA^{Arg}_{UCU}$, which is the only $tRNA^{Arg}$ isoacceptor that shows high levels of misacylation (Figure1C). These results indicate that the misacylated tRNA species are used in translation.

tRNA mismethionylation with purified yeast components *in vitro*

We used recombinant, purified yeast AME complex from *E. coli* to demonstrate that this complex is sufficient to mismethionylate yeast tRNAs (Figure 5). *In vitro* aminoacylation with *S. cerevisiae* $tRNA^{Met}$ previously used yeast MetRS alone in the presence or absence of Arc1p, not the full AME complex (10,32). To better recapitulate cellular conditions, we used the AME complex for all of our *in vitro* aminoacylation studies. The reaction scheme involves incubating the purified AME complex with total yeast tRNA with ^{35}S-Met, followed by hybridization of the reaction mixture on the microarray (Figure 5A). Our affinity purified AME complex was derived from an overexpression plasmid in *E. coli*; it contains an amino-terminal His_6 tag on the Arc1p protein. Since the His_6 tag is only on the Arc1p protein, excess Arc1p appears to be purified with the

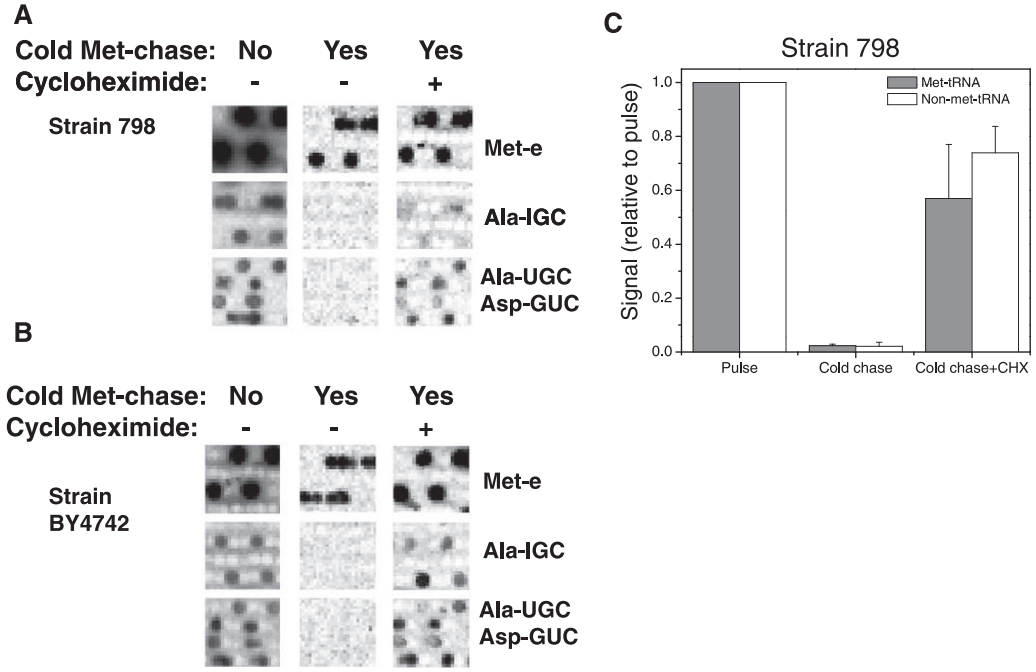


Figure 3. Mismethionylated tRNAs have similar cellular utilization kinetics as $tRNA^{Met}$s. Yeast strains 798 (**A**) or BY4742 (**B**) were pulse-labeled with ^{35}S-Met for 1 min followed by 1 min cold chase with a large excess of Met with or without cycloheximide. We used two strains to indicate that the cellular utilization kinetics of mismethionylated tRNAs are not strain-specific. Strain 798 is fully prototrophic, whereas BY4742 is the parent strain from where all isogenic deletion strains are derived. (**C**) Quantitative comparison of the disappearance of Met-tRNA and non-Met-tRNA signals in the absence of cycloheximide or the maintenance of Met-tRNA and non-Met-tRNA signals in the presence of cycloheximide in strain 798.

complex during Ni-NTA affinity purification, as seen by SDS-PAGE (Figure 5B). As expected, essentially all synthetase molecules are associated with Arc1p, and the majority of both synthetases form a single peak in size exclusion chromatography (Figure 5C).

We charged total RNA isolated from stationary phase yeast with ^{35}S-Met using the purified AME complex (Figure 5D). Many tRNA species were misacylated and mis-methionylation increases over time (Figure 5E). All the misacylated tRNA species *in vitro* were also misacylated *in vivo*. The greater extent of misacylation *in vivo* may be due to the AME complex association with polysomes, although this remains to be determined. The mammalian multi-synthetase complex is associated with polysome (33), and the polysome-associated multi-synthetase complex misacylates more tRNA species *in vitro* compared to the multi-synthetase complex alone (4). When the *in vitro* charging sample was first deacylated at pH 9 followed by array hybridization, no deacylation-resistant tRNA was observed, as expected due to the lack of post-translational modification enzymes in the *in vitro* charging mixture (Figure 5E). Quantification of the *in vitro* misacylated tRNA species showed high levels for tRNA species mostly coding for charged and polar amino acid side chains (Figure 5D). Although a very extensive pattern of *in vitro* misacylation is present, still more mismethionylated tRNA species are observed *in vivo* than *in vitro*. Similar results have been seen for tRNA mismethionylation in mammalian systems (4).

The Arc1p in the AME complex is a generic tRNA-binding protein and assists tRNA binding to the MetRS and GluRS for aminoacylation (10,14). The extensive level of misacylated tRNA species in *S. cerevisiae* suggests that Arc1p may also shuttle non-Met-tRNAs to the MetRS to be aminoacylated with methionine. We performed an experiment to compare tRNA binding versus mis-methionylation by the AME complex (Figure 6). We first incubated 5′- ^{32}P-labeled total yeast tRNA with the purified AME complex at varying concentrations of KCl followed by affinity pull down of the AME complex. Bound ^{32}P-labeled tRNAs were then examined by micro-array (Figure 6A and B). Almost all detectable tRNA species are bound by AME at 0.1 M KCl; increasing KCl to 0.4 M reduced the number of tRNA species bound to the complex as expected (Figure 6C). This result is consistent with the ability of AME to bind essentially any tRNA (32). Among the bound tRNAs at 0.1 M KCl, only a subset is mismethionylated, suggesting that mis-methionylation has additional requirement beyond simple binding.

Finally, we applied the classical filter-based aminoacylation assays to confirm that the AME complex is capable of misacylating unmodified tRNA transcripts at high efficiency (Figure 7). We chose to work with transcripts of $tRNA^{Met}_e$ (Figure 7A, left) and variants of $tRNA^{Glu}$ (Figure 7A, middle) because the AME contains both MetRS and GluRS. The reference yeast genome contains two $tRNA^{Glu}$ isoacceptors: 2 copies of $tRNA^{Glu}_{CUC}$ and 13 copies of $tRNA^{Glu}_{UUC}$. The $tRNA^{Glu}_{UUC}$ isoacceptor family has two isodecoders, a major form with 12 copies and a minor form with a single copy (16,34). The $tRNA^{Glu}_{UUC(12)}$ and $tRNA^{Glu}_{UUC(1)}$ isodecoders differ by one nucleotide in

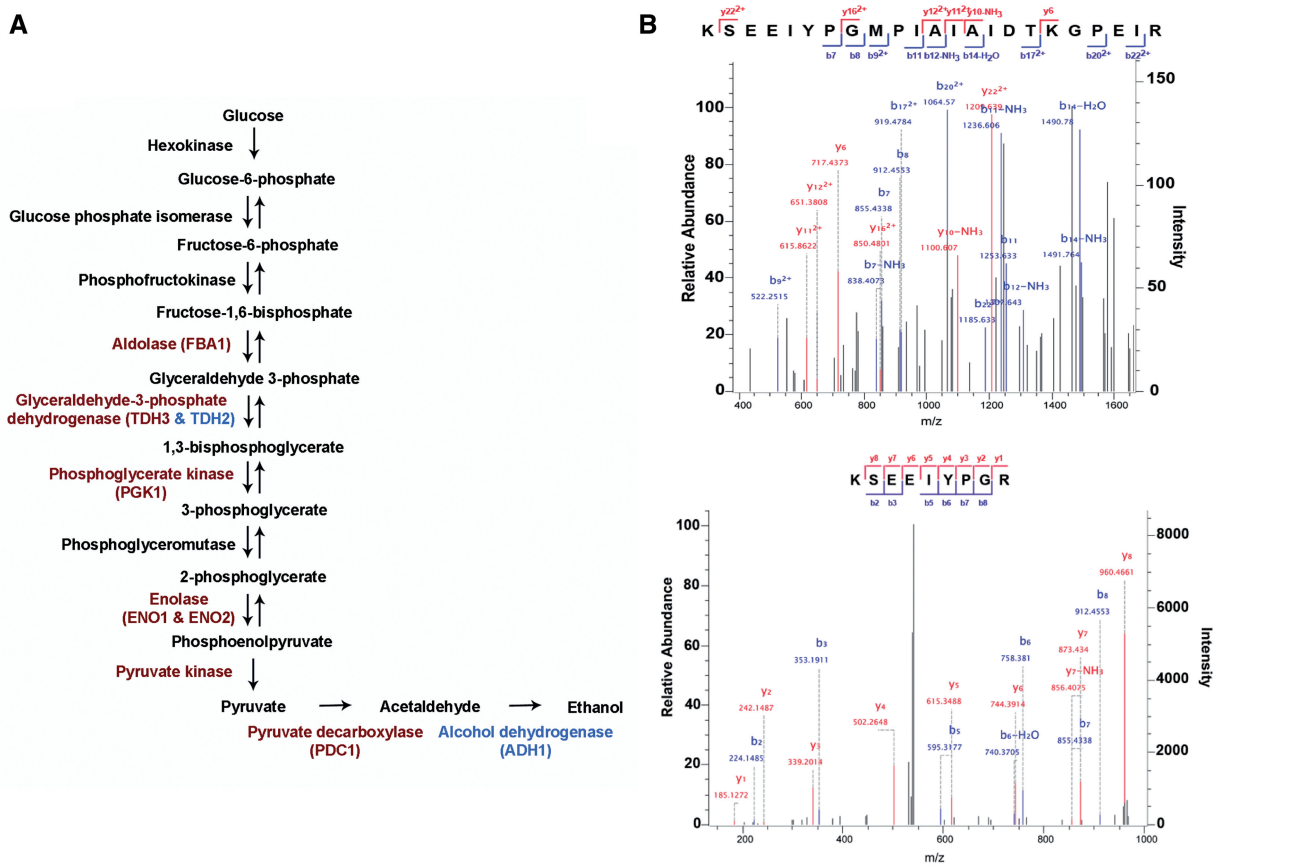


Figure 4. Mismethionylated tRNAs are used in translation. (**A**) Glycolysis pathway showing the nine proteins analyzed in this study from a proteomic mass spectrometry dataset. These nine proteins were chosen based on their high occurrence in this mass spec dataset. For simplicity, only Lys/Arg→Met substitutions were searched for in our analysis. Thirty-one peptides from a total of 4675 peptides identified are derived from K/R→M substitutions (0.66%). Proteins with (maroon) or without (blue) detected mistranslated peptides are shown. (**B**) Spectra for one example of a mistranslated peptide (top) and its wild-type peptide (bottom) in CDC19, the pyruvate kinase. The AGA codon at this mistranslated position should be read by $tRNA^{Arg}_{UCU}$, which is the only $tRNA^{Arg}$ isoacceptor misacylated with Met. As expected, the relative abundance of the mistranslated peptide is significantly lower compared to the wild-type peptide (Y-axis scale on the right).

their acceptor stems, and the isoacceptor $tRNA^{Glu}_{CUC}$ differs by five nucleotides from $tRNA^{Glu}_{UUC(12)}$. These three $tRNA^{Glu}$s were not distinguishable on our microarrays due to their sequence similarities. We also made three $tRNA^{Glu}$ mutants (Figure 7A, right) in the acceptor stems and anticodon loop to probe possible sequence identity elements of misacylation.

The purified AME complex charged all the tRNA transcripts with their cognate amino acids, $tRNA^{Met}_{e}$ with methionine or all $tRNA^{Glu}$s with glutamic acid (Figure 7B). All three $tRNA^{Glu}$ variants as well as all three $tRNA^{Glu}$ mutants were mismethionylated at similar levels under this reaction condition. As a positive control, very little mischarging of $tRNA^{Met}_{e}$ with Glu is present, showing that misacylation occurs exclusively by the MetRS (Figure 7B). This result suggests that all three $tRNA^{Glu}$ variants could have contributed to the total ^{35}S-signal for the $tRNA^{Glu}$ on our microarray. We also measured the charging kinetics of $tRNA^{Met}_{e}$ and the major form of $tRNA^{Glu}$ with Met or Glu (Figure 7C). A two-phase charging kinetics was observed in all cases. Both phases have essentially the same K_m values, but they differ by over 100-fold in k_{cat} values. The reason for such two-phase charging behavior by the purified AME complex is unclear. However, both the K_m and the k_{cat} values for the fast phase is within the same order of magnitude observed previously, although all previous experiments were performed with just the MetRS or GluRS protein not the AME complex. The AME charged $tRNA^{Met}_{e}$ with Met at k_{cat}/K_m of $0.39\,\mu M^{-1}s^{-1}$ and $tRNA^{Glu}_{UUC(12)}$ with Glu at k_{cat}/K_m of $1.1\,\mu M^{-1}s^{-1}$, indicating that our recombinant AME is highly active. The AME mischarged $tRNA^{Glu}_{UUC(12)}$ with Met at k_{cat}/K_m of $0.2\,\mu M^{-1}s^{-1}$, which is only 2-fold lower than cognate charging. This result is significantly different from *in vitro* mischarging of the AME complex using total yeast tRNA (Figure 5D), which suggests that certain modifications in $tRNA^{Glu}$ may significantly reduce mismethionylation.

DISCUSSION

We have demonstrated here that tRNA misacylation with methionine occurs in *S. cerevisiae*. This represents the second example of tRNA mismethionylation in cells in addition to mouse and humans. Since yeast is

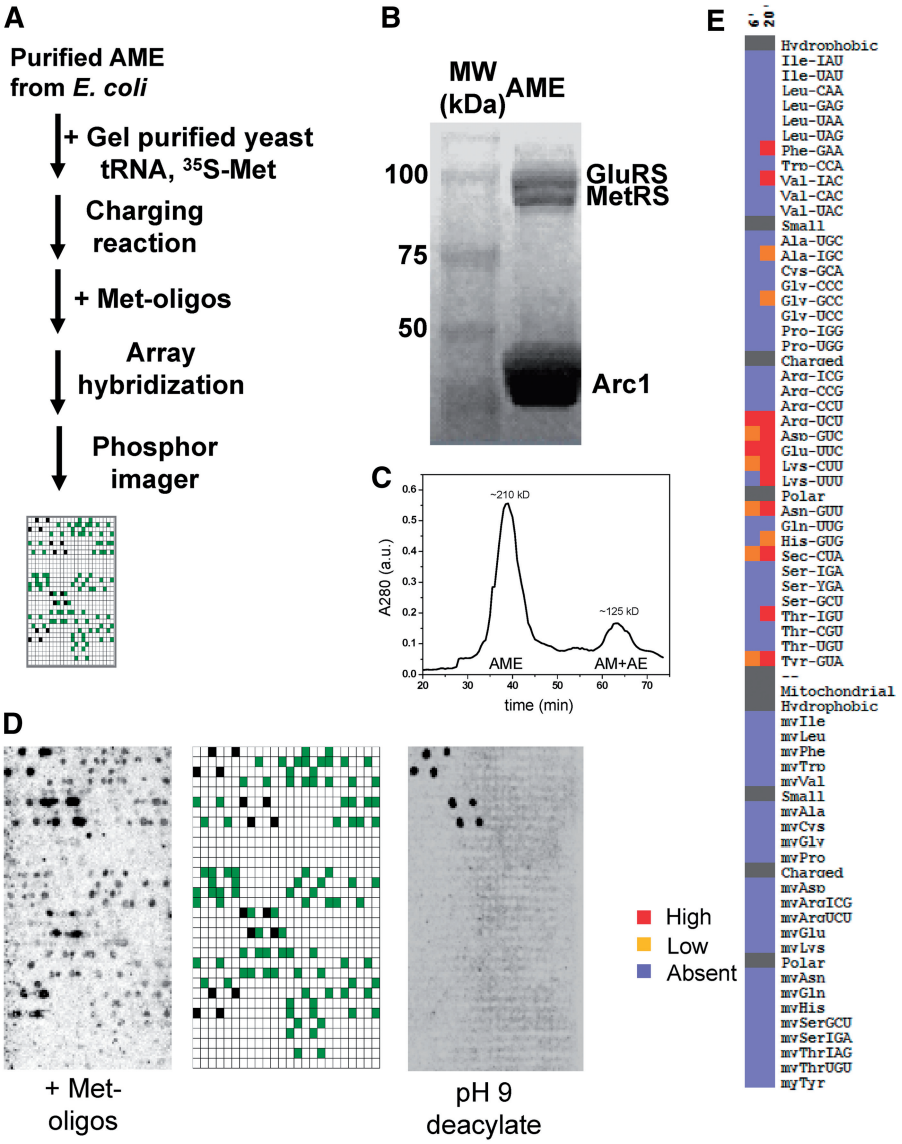


Figure 5. Mismethionylation of yeast tRNAs by recombinant AME *in vitro*. (**A**) Experimental flow. (**B**) SDS-PAGE analysis of purified recombinant AME from *E. coli*. The complex is His_6-tagged at Arc1p, which explains a possible over-representation of the Arc1p protein in this preparation. (**C**) Size exclusion chromatography using a gel filtration Superdex 200 column shows that the AME complex contains both synthetases associated with Arc1p. (**D**) Total, deacylated tRNA isolated from stationary phase yeast strain 798 was gel purified and then charged with ^{35}S-Met using purified AME. The RNA from 20 min charging was hybridized to the array in the presence of excess free $tRNA^{Met}$ probes. The array layout shows cytosolic Met-tRNA probes as black squares and non-Met-tRNA probes showing misacylation as green squares. Following 20 min charging, another sample was deacylated and hybridized to the array. The deacylation resistant signals from the cellular yeast samples are absent from the array as expected. The remaining signal is derived from incompletely deacylated $tRNA^{Met}_e$. (**E**) Quantification of *in vitro* results showing more misacylation occurs after 20′ than 6′.

evolutionarily distant from mammals, this result suggests that tRNA mismethionylation is conserved from fungi to mammalian lineages. We also show that tRNA mismethionylation is derived from the activity of the MetRS. Mismethionylation occurs *in vitro* when the yeast MetRS is associated with the other two proteins in the AME complex. This association in the complex, however, is not required for mismethionylation *in vivo* as an *Arc1* deletion yeast strain also shows similar level of mismethionylation. We further show by *in vivo* utilization kinetics and proteomic mass spec analysis that misacylated tRNAs are used in translation in yeast, as in mammals (4).

Our results lead to two wide-open biological questions. First, how do ribosome choose mismethionylated tRNAs in translation? Misacylated tRNA may or may not be used in translation depending on elongation factor selection and ribosome utilization. In *E. coli*, misacylated tRNAs can bind to the elongation factor EF-Tu at different affinities compared to correctly charged tRNAs (30,35). This differential binding has been shown to result in the exclusion of some misacylated tRNAs to EF-Tu binding while some other misacylated tRNAs cannot be properly delivered into the A-site of the ribosome (31). How mismethionylated tRNAs are selected by EF-Tu is, however, unclear as the EF-Tu selection of misacylated

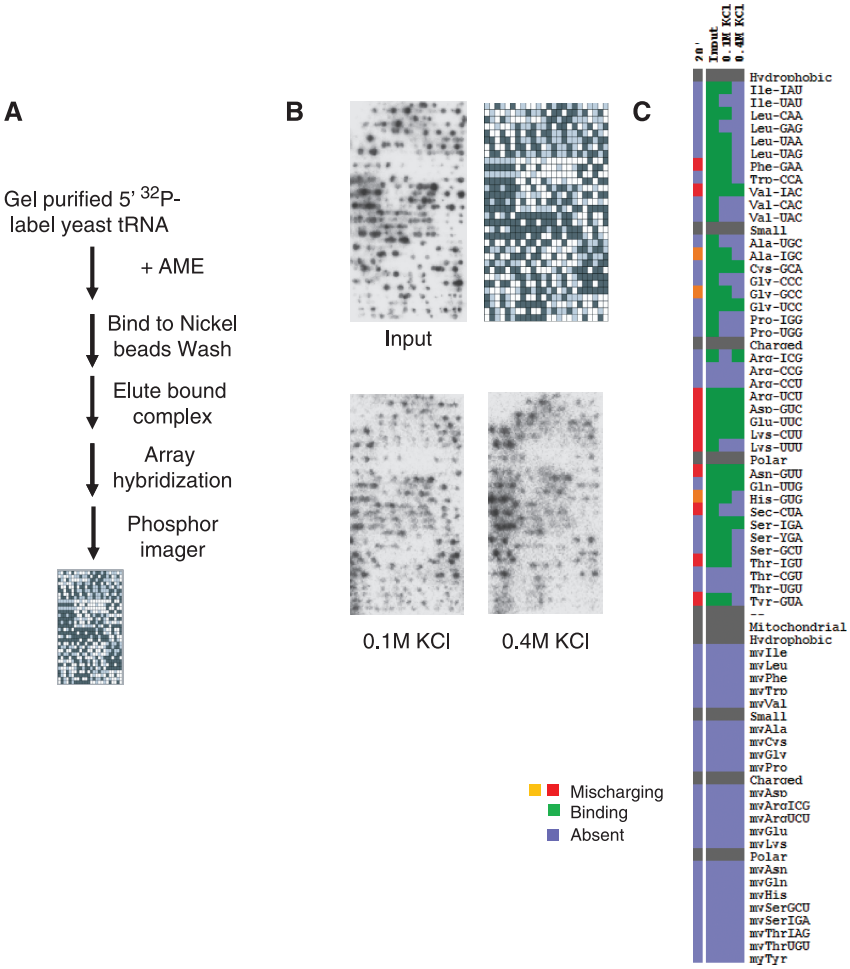
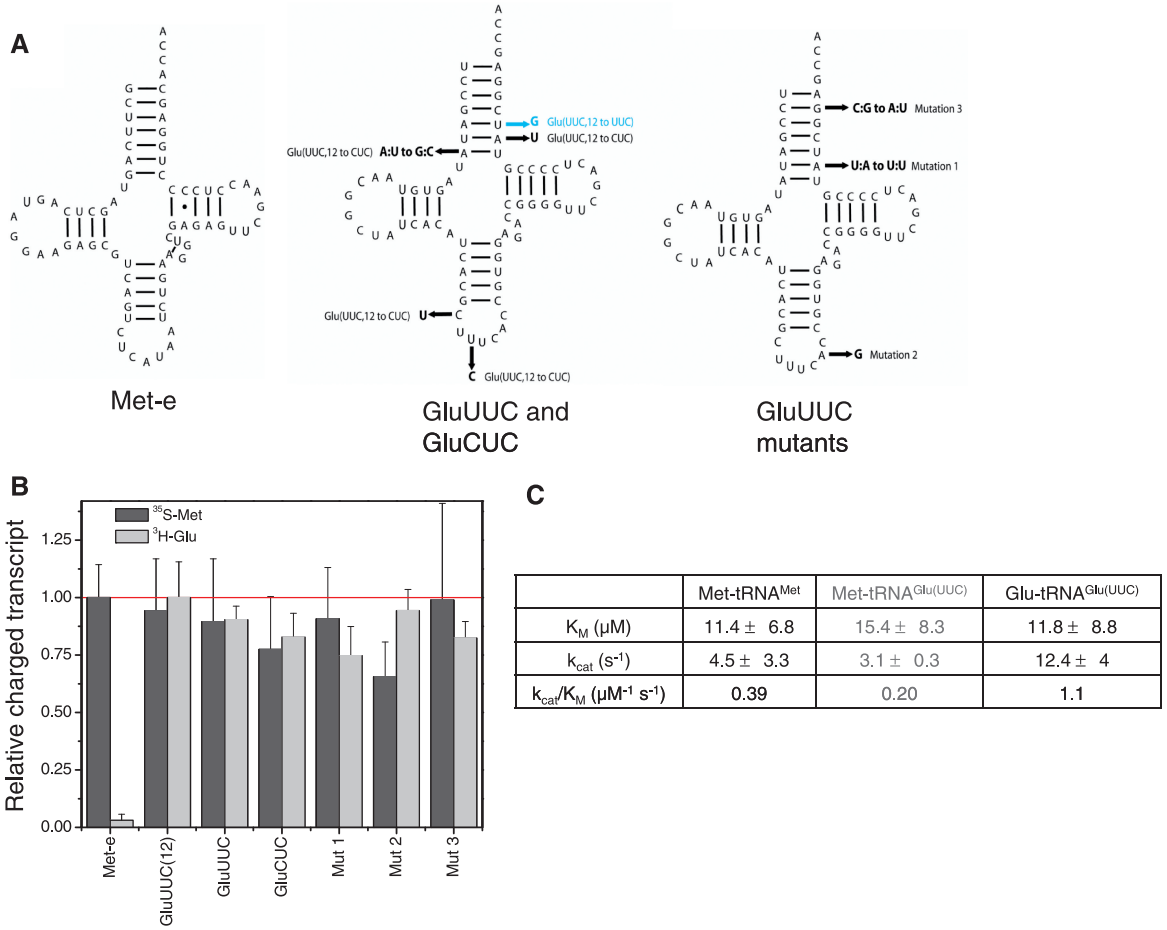


Figure 6. tRNA binding to the AME complex *in vitro*. (**A**) Experimental flow. (**B**) Purified yeast tRNA was 5′-^{32}P-labeled and incubated with AME at varying concentrations of KCl. Arrays show the total tRNA input and tRNAs eluted from AME in the presence of 0.1M KCl or 0.4M KCl. The array layout shows all cytosolic tRNA probes as black squares and all mitochondrial tRNA probes as light gray squares. (**C**) Comparing *in vitro* misacylation to tRNA binding to AME. All mismethionylated tRNAs *in vitro* bind to AME at 0.1 M KCl.

tRNAs depends on the tRNA and the amino acid identity; mismethionylated tRNAs were not used in previous studies. In the fungal CTG clade species, the tRNA with anticodon CAG is charged with either serine or leucine, and both Ser and Leu charged tRNAs are used in translation (36). This is one example of experimental evidence for the utilization of mischarged tRNAs in eukaryotes, suggesting that EF-1α does not vigorously discriminate misacylated tRNAs. Many studies have been conducted on how ribosome discriminates codon-anticodon mismatched tRNAs in the A and the P sites. Mismethionylated tRNAs, however, can enter the A site while maintaining perfect codon–anticodon matches. For instance, a mismethionylated $tRNA^{Lys}_{CUU}$ is expected to enter the A site containing the cognate AAG codon. We do not know how mismethionylated tRNA in the A site might perturb peptide bond formation or translocation, due to a lack of previous studies that specifically considers mismethionylated tRNAs.

Although EF-1α may not discriminate misacylated tRNAs, our proteomic analysis here shows that misacylated lysyl- and argininyl-tRNAs are used in translation. To identify the rules of ribosome utilization of mismethionylated tRNAs in the future, it should be possible to conduct proteomic studies to specifically identify Met substitutions in proteins that can be considered to derive from all the mismethionylated tRNA species. Each mismethionylated tRNA species is only present at an average level of ~0.5% of $tRNA^{Met}$s. If we consider that ribosome uses all such tRNAs, it will still represent a sub-1% presence of Met at individual non-Met positions in proteins. Met-containing peptides are also prone to oxidation in mass spectrometry analysis, which pose an additional challenge for proteome-wide identification. Unlike Cys-containing peptides, which can be specifically enriched from the proteomic mixture, no reliable chemical method is yet available to enrich Met-containing peptides.

The second biological question deals with the potential function of mis-translation using mismethionylated tRNAs. We have proposed previously for mammalian cells that low-frequency substitution of non-Met residues with Met in stress response proteins can enhance the known, protective function of genetically encoded



	Met-tRNA^{Met}	Met-tRNA$^{Glu(UUC)}$	Glu-tRNA$^{Glu(UUC)}$
K_M (μM)	11.4 ± 6.8	15.4 ± 8.3	11.8 ± 8.8
k_{cat} (s^{-1})	4.5 ± 3.3	3.1 ± 0.3	12.4 ± 4
k_{cat}/K_M (μM^{-1} s^{-1})	0.39	0.20	1.1

Figure 7. Mismethionylation of tRNA^{Glu} transcripts. All tRNA^{Glu} transcripts were prepared by first making T7 transcripts containing a 5′-leader. The 5′-leader was then removed by RNase P cleavage to produce mature tRNA^{Glu} containing U+1. (**A**) Sequence of the mature tRNA$^{Met}_e$ and tRNA^{Glu} transcripts. Left: tRNA$^{Met}_e$; middle: tRNA^{Glu} from the reference yeast genome; right: tRNA^{Glu} mutants derived from the major tRNA$^{Glu}_{UUC(12)}$. (**B**) Relative saturation charging after 20 min of tRNA$^{Met}_e$ and tRNA^{Glu} transcripts with Met or Glu using the purified AME. All tRNA^{Glu} transcripts and mutants were mischarged with Met. However, tRNA^{Met} was not mischarged with Glu. (**C**) K_m, k_{cat} and k_{cat}/K_m values for the fast phase of tRNA^{Met} charging with Met (Met-tRNA$^{Met}_e$), tRNA^{Glu} charging with Met (Met-tRNA$^{Glu}_{UUC}$) and tRNA^{Glu} charging with Glu (Glu-tRNA$^{Glu}_{UUC}$).

Met residues against ROS inactivation of cells' own proteins. ROS refers to a collection of highly reactive radicals or peroxides, byproducts of the electron transport chain, and as such used as signaling molecules for cell health and stress (37). In mammals, ROS is also used as chemical weapons against invading microbes or undesired molecules. Their high chemical reactivity easily leads to damages of a cell's own molecules, including proteins. To protect their own proteins against ROS inactivation, certain Met residues in an endogenous protein are positioned at strategic places to react first with diffusing ROS molecules before they can oxidize sensitive amino acid side chains in, e.g. an active site of an enzyme to result in permanent inactivation (5,38). Our previous proposal suggests that substituting certain non-Met residues with Met during translation can enhance this effect, in particular, during oxidative stress response. Our yeast result here is consistent with this idea in that many tRNA encoding charged and polar amino acids are mismethionylated so that a Met substitution at these residues would enable such a protective function but less likely produce misfolded proteins. In order to test this functional proposal for mis-translation using mismethionylated tRNAs, one would need MetRS mutants that have diminished ability to mismethionylate but still maintain a wild-type level activity to charge tRNA^{Met}. Such MetRS mutants are available for the *E. coli* enzyme (7), and we are actively identifying such mutants using the recombinant AME as the starting point.

To rule out misacylation caused by other enzymes in yeast cells, we purified the recombinant AME complex from *E. coli* and performed *in vitro* aminoacylation reactions with purified total yeast tRNA and tRNA transcripts. Mismethionylation was prevalent for many tRNAs and for all tRNA^{Glu} transcripts tested. All the misacylated tRNAs *in vitro* are also misacylated *in vivo*, suggesting that the AME complex binds many tRNAs and facilitates interaction of many tRNAs with the MetRS for methionylation. It appears that the Arc1p protein in the AME complex facilitates interactions of MetRS with tRNA^{Met} and a large number of other tRNAs. However, Arc1p is not required for methionylation *in vivo*.

The *E. coli* MetRS misacylates only two *E. coli* tRNAs, $tRNA^{Arg}_{CCU}$ and $tRNA^{Thr}_{CGU}$ *in vitro* (7). These two tRNAs have anticodons that differ from the anticodon for methionine, CAU, by one nucleotide. The anticodon and several regions of the *E. coli* MetRS that interact with the anticodon loop are responsible for misacylation. In addition, several studies have shown that the CAU anticodon plays a key role in yeast tRNA methionylation and discrimination (39). As there are a large number of different misacylated tRNAs in *S. cerevisiae*, the misacylated tRNAs have little similarity among their anticodons. This raised the question of how so many different tRNAs are misacylated with methionine in *S. cerevisiae*. We found here that many tRNA species bind to the AME complex, including all the tRNAs that are misacylated. Since Arc1p is a generic tRNA-binding protein, Arc1p can bind many tRNAs and likely transfer them to the MetRS for aminoacylation with methionine, potentially explaining why the AME complex misacylates many tRNA species *in vitro*.

In summary, tRNA mismethionylation occurs in mammals and as shown here in yeast. As in mammals, a large number of tRNAs can be mismethionylated in yeast, and these misacylated tRNAs are used in translation. The misacylated tRNAs code for charged or polar amino acids, corroborating a role for Met substituting more solvent exposed positions in proteins. This work provides another role of multisynthetase complexes in eukaryotes and expands our knowledge on the mystery of molecular and biological roles of mis-translation.

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